PART I - ADMINISTRATIVE

Section 1. General administrative information

Title of project

Endocrine Control Of Ovarian Development In Salmonids

BPA project number: 20044
Contract renewal date (mm/yyyy): 10/2000 ☒ **Multiple actions?**

Business name of agency, institution or organization requesting funding
University of Idaho

Business acronym (if appropriate) UI

Proposal contact person or principal investigator:

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NPPC Program Measure Number(s) which this project addresses
7.2; 7.4D

FWS/NMFS Biological Opinion Number(s) which this project addresses

Other planning document references

Short description
Study key intra-ovarian endocrine pathways in salmonids as a means to address reproductive problems in captive broodstock programs. Provide basis for technological development to diagnose sub-fertility and increase embryo viability.

Target species
Oncorhynchus mykiss (steelhead/rainbow trout)

Section 2. Sorting and evaluation

Subbasin
Systemwide

Evaluation Process Sort

CBFWA caucus	Special evaluation process	ISRP project type

Mark one or more caucus	If your project fits either of these processes, mark one or both	Mark one or more categories
☒ Anadromous fish ☐ Resident fish ☐ Wildlife	☒ Multi-year (milestone-based evaluation) ☐ Watershed project evaluation	☐ Watershed councils/model watersheds ☐ Information dissemination ☐ Operation & maintenance ☐ New construction ☒ Research & monitoring ☐ Implementation & management ☐ Wildlife habitat acquisitions

Section 3. Relationships to other Bonneville projects

Umbrella / sub-proposal relationships. List umbrella project first.

Project #	Project title/description

Other dependent or critically-related projects

Project #	Project title/description	Nature of relationship
	Intracytoplasmic sperm injection: genetic retrieval from single sperm	Fish Reproduction Program participant
	Analyzing genetic and behavioral changes during salmonid domestication	Fish Reproduction Program participant
	Induction of precocious sexual maturity and enhanced egg production in fish	Fish Reproduction Program participant
	Enhancement of salmonid gamete quality by manipulation of intracellular ATP	Fish Reproduction Program participant; co-investigator
	Viral vaccines and effects on reproductive status	Fish Reproduction Program participant

Section 4. Objectives, tasks and schedules

Past accomplishments

Year	Accomplishment	Met biological objectives?

Objectives and tasks

Obj 1,2,3	Objective	Task a,b,c	Task
1	determine an intra-ovarian endocrine role for estradiol	a	set up and optimize methods to quantify salmonid IGF mRNA and protein
		b	test whether estradiol can influence IGF

			mRNA and/or protein levels within the ovary
2	promote ovarian follicle growth	a	test whether estradiol, or other endocrine factors produced within the ovary, can increase ovarian growth
3	influence the transcription of oocyte vitellogenin receptors	a	develop suitable oocyte vitellogenin receptor mRNA probes
		b	test the ability of intra-ovarian endocrine factors (i.e. estradiol, IGFs) to modulate vitellogenin receptor mRNAs

Objective schedules and costs

Obj #	Start date mm/yyyy	End date mm/yyyy	Measureable biological objective(s)	Milestone	FY2000 Cost %
1	10/1999	10/2000	experimental data showing the effects of estradiol on in vitro IGF mRNA and/or protein synthesis by the salmonid ovary		33.30%
2	10/1999	10/2000	hormonal enhancement of ovarian growth		33.30%
3	10/1999	10/2000	upregulation/ downregulation of oocyte vitellogenin receptor transcripts by the intra-ovarian endocrine agents tested		33.30%
					0.00%
					0.00%
				Total	99.90%

Schedule constraints
None

Completion date
10/2004

Section 5. Budget

FY99 project budget (BPA obligated): $ 0

FY2000 budget by line item

Item	Note	% of total	FY2000
Personnel	salaries: PI (2 mo); research technician and postdoctoral fellow (12 mo)	%33	73,560
Fringe benefits	28.5% for PI and professional staff	%9	20,965
Supplies, materials, non-expendable property	consumables, reagents, plumbing supplies, fish	%6	13,000

Operations & maintenance	equipment repair and maintenance	%1	5,000
Capital acquisitions or improvements (e.g. land, buildings, major equip.)	water chillers, recirculating pumps, experimental tanks	%4	10,000
NEPA costs			0
Construction-related support			0
PIT tags	# of tags:		0
Travel	annual professional meeting	%0	2,000
Indirect costs	UI rate of 44.7% on total direct costs	%30	68,625
Subcontractor			0
Other	aquaculture core facility; hormone core facility; administrative core facility	%13	29,000
		TOTAL BPA FY2000 BUDGET REQUEST	$222,150

Cost sharing

Organization	**Item or service provided**	**% total project cost (incl. BPA)**	**Amount ($)**
None			
		Total project cost (including BPA portion)	$222,150

Outyear costs

	FY2001	**FY02**	**FY03**	**FY04**
Total budget	$201,700	$209,033	$216,732	$224,815

Section 6. References

Watershed?	**Reference**
☐	Barr, W.A. 1963. The endocrine control of the sexual cycle in the plaice, Pleuronectes platessa (L). II. The endocrine control of oogenesis. Gen. Comp. Endocrinol. 3: 205-215.
☐	Blaxter, J.H.S., 1988. Pattern and variety in development. In: Fish Physiology, (Hoar and Randall, eds.) Vol. XI, The Physiology of developing fish, Part A, Eggs and Larvae. Academic, San Diego, pp. 1- 58.
☐	Cao, Q.-P., S.J. Duguay, E. Plisetskaya, D.F. Steiner and S.J. Chan. 1989. Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor I mRNA. Mol. Endocrinol. 3: 2005-2010.
☐	Chen, T.T., A. Marsh, M. Shamblott, K.-M. Chan, Y.-L. Tang, C.M. Cheng and B.-Y. Yang. 1994. Structure and evolution of fish growth hormone and insulinlike growth ... In: Fish Physiology, (Hoar and Randall, eds.) Vol. XIII, pp.179-203. Academic Press, NY.
☐	Das, S.K., H. Tsukamura, B.C. Paria, G.K. Andrews and S.K. Dey.1994. Differential expression of epidermal growth factor receptor (EGR-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse … Endocrinology 134:971-981.
☐	Davidson, E.H. 1986. Gene activity in early development. Academic Press, Inc. Orlando, FL.
☐	Dorrington, J. H., J.J. Bendell, and S.A. Khan. 1993. Interactions between FSH, estradiol-17-beta and transforming growth factor-beta regulate growth and differentiation in the rat gonad. J. Steroid Biochem. Mol. Biol. 44: 441- 449.

☐	Duguay, S.J., L.K. Park, M. Samadpour, and W.W. Dickhoff. 1992. Nucleotide sequence and tissue distribution of three insulin-like growth factor I prohormones in salmon. Mol. Endocrinol. 6: 1202-1210.
☐	Hsu, C.-J. and J.M. Hammond. 1987. Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro. Endocrinology 120: 198-207.
☐	Kagami, I., H. Mizunuma, S. Miyamoto, Y. Ibuki, and T. Uchida. 1996. Quantification of estrogen receptor messenger RNA by quantitative polymerase chain reaction using internal standard fragment. Biochem. Biophys. Res. Com. 228: 358-364.
☐	Maestro, M.A., E. Mendez, M. Parrizas, and J. Gutierrez. 1997. Characterization of insulin and insulin-like growth factor-1 ovarian receptors during the reproductive cycle of carp (Cyprinus carpio). Biol. Reprod. 56: 1126-1132.
☐	Manova, K., E.J. Huang, M. Angeles, V. De Leon, S. Sanchez, S.M. Pronovost, P. Besmer, and R.F. Bachvarova. 1993. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte … Dev. Biol. 157: 85-99.
☐	Nagahama, Y., M. Yoshikuni, M. Yamashita, and M. Tanaka. 1994. Regulation of oocyte maturation in fish. In: Fish Physiology, (Hoar, Randall, and Farrell, eds.) Vol. XIII, pp. 331-357. Academic Press, New York.
☐	Nagler, J.J., A.P. Scott, C.R.Tyler and J.P. Sumpter. 1996. Gonadotropins I and II do not stimulate the in vitro secretion of 17a,20b-dihydroxy-4-pregnen-3-one-20-sulphate by rainbow trout gonads during final sexual … Fish Physiol. Biochem.15: 149-156.
☐	Nagler, J.J., C.R. Tyler and J.P. Sumpter. 1994. Ovarian follicles of rainbow trout (Oncorhynchus mykiss) cultured within lamellae survive well, and sequester and process vitellogenin. J. Exp. Zool. 269:45-52.
☐	Nagler, J.J., and D.G. Cyr. 1997. Exposure of male American plaice (Hippoglossoides platessoides) to contaminated marine sediments decreases the hatching success of their progeny. Environ. Tox. Chem. 16: 1733-1738.
☐	Nagler, J. J. and D.G. Cyr. 1998. Different gonadal estrogen receptor messenger RNAs in the rainbow trout: is this ER-a and ER-b? Biol. Reprod. 58: Suppl. 1, Abstract No. 232.
☐	Negatu, Z., S.M. Hsiao, and R.A.Wallace. 1998. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in Fundulus heteroclitus. Fish Physiol. Biochem. 19:13-21.
☐	Packer, A.I., Y. Hsu, P. Besmer, and R.F. Bachvarova. 1994. The ligand of the c-kit receptor promotes oocyte growth. Dev. Biol. 161: 194-205.
☐	Prat, F., J.P. Sumpter, C.R. Tyler. 1996. Validation of radioimmunoassays for two salmon gonadotropins (GtH I and GtH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout. Biol. Reprod. 54: 1375-1382.
☐	Prat, F., K. Coward, J.P. Sumpter, and C.R. Tyler. 1998. Molecular characterization and expression of two ovarian lipoprotein receptors in the rianbow trout (Oncorhynchus mykiss). Biol. Reprod. 54: 1375-1382.
☐	Pratt, W.B. and L.C. Scherrer. 1993. Heat shock proteins and the cytoplasmic-nuclear trafficking of steroid receptors. In Steroid Hormone Receptors: Basic and Clinical Aspects, V.K. Moudgil, Ed., Birkhauser Boston, pp. 215- 246.
☐	Richards, J. 1994. Hormonal control of gene expression in the ovary. Endocrine Rev. 15:725-751.
☐	Shamblott, M.J. and T.T. Chen. 1992. Identification of a second insulin-like growth factor in a fish species. Proc. Natl. Acad. Sci. USA 89:8913-8917.
☐	Suzuki, K., Y. Nagahama, and H. Kawauchi. 1988. Steroidogenic activities of two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71: 452-458.
☐	Swanson, P., T. Flagg, J. Hard, L. Harrell, K. Shearer, R. Pascho, W. Hershberger, K. Massey, and R. Hardy. 1995. Research on captive broodstock technology for Pacific salmon. Annual Report 1995. DE-AI79-93BP55064, BPA, Portland, OR.
☐	Tyler, C.R. and P. Lancaster. 1993. Isolation and characterizaton of the receptor for vitellogenin from follicles of the rainbow trout, Oncorhynchus mykiss. J. Comp. Physiol. B

	163: 225-233.
☐	Tyler, C.R., J. J. Nagler, T. G. Pottinger and M. A. Turner. 1994. Effects of unilateral ovariectomy on recruitment and growth of follicles in the rainbow trout, Oncorhynchus mykiss. Fish Physiol. Biochem. 13:309-316.
☐	Tyler, C.R., T.G. Pottinger, K. Coward, F. Prat, N. Beresford, and S. Maddix. 1997. Salmonid follicle-stimulating hormone (GTH I) mediates vitellogenic development of oocytes in the rainbow trout, Oncorhynchus mykiss. Biol. Reprod. 57:1238-1244.
☐	Yan, L., P. Swanson and W.W. Dickhoff. 1992. A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biol. Reprod. 47:418-427.
☐	Wallace, R.A. 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: L.W. Browder (Ed) Developmental biology, Vol.1., Plenum Press, New York, pp. 127-177.
☐	Zafarullah, M., J. Wisniewski, N.W. Shworak, S. Schieman, S. Misra and L. Gedamu. 1992. Molecular cloning and characterization of a constitutively expressed heat-shock-cognate hsc7gene from rainbow trout. Eur. J. Biochem. 204: 893-900.

PART II - NARRATIVE

Section 7. Abstract

Reduced female reproductive performance is currently limiting the number of viable offspring produced by captive salmonid broodstock programs in the Pacific Northwest. The consequences of these difficulties is an uncertain supply of quality eggs for artificial propagation, and endangerment of certain populations where the number of remaining females is small. An explanation for some instances of sub-fertility and variable embryo survival can undoubtedly be linked to oogenesis and the endocrine events that regulate oocyte development. To date, our understanding of intra-ovarian endocrine physiology in salmonids is limited, but research in this area could make an important contribution toward improving reproductive success. The discovery, in my laboratory, of estrogen receptor transcripts in the rainbow trout (*Oncorhynchus mykiss*) ovary raises the possibility that estrogens have important effects within the ovary that relate directly to oocyte development. **The general hypothesis of this proposal is that an understanding of key endocrine processes within the developing salmonid ovary, particularly those involving estradiol (E_2), will lead to ways of diagnosing sub-fertility and improving reproductive success.** The specific objectives are to, 1) establish whether the principal female sex steroid, E_2, can stimulate insulin-like growth factor production in the ovary, 2) determine whether E_2, or hormones stimulated by E_2, can promote the growth and development of ovarian follicles, and 3) study whether E_2 can influence the transcription of genes for vitellogenin receptors within developing oocytes. The outcome of these studies are two-fold, first, it will generate basic information to understand what controls the normal growth of ovarian follicles, and secondly, to provide a basis for future strategies and technologies to improve salmonid fecundity and embryo viability.

Section 8. Project description

a. Technical and/or scientific background

The ideal outcome of successful ovarian development in fishes is the production of quality eggs that will yield viable offspring (Blaxter, 1988). Until recently it was not clear in salmonids how the contributions of the female and male gametes affected embryo viability, but it has been shown in rainbow trout (*Oncorhynchus mykiss*) that, once fertilized, the egg (i.e. female component) is critical for successful early embryogenesis to the point of swim-up (Nagler, preliminary studies). The male contribution to early embryonic survival is negligible. Therefore, the quality of the salmonid egg and resulting viability of the embryo is tied to the female. The specific reasons for sub-fertility, in instances of variable embryo survival, are largely unknown but the varied events that occur during oocyte development likely hold the key to understanding these problems.

In egglaying fishes, oogenesis is characterized by a lengthy period during which the oocyte is arrested in prophase I of meiosis (Nagahama et al. 1994). During this period of meiotic arrest the ovary undergoes essentially all of its growth, in terms of follicle cell proliferation and oocyte enlargement, in preparation for final maturation. Two different developmental phases are delineated during the growth period, an initial pre-vitellogenic phase, followed by a vitellogenic phase. The discrimination between these two phases has to do with the start of yolk accumulation within the cytoplasm of the oocyte, which marks the end of the pre-vitellogenic phase and the start of the vitellogenic phase. The pre-vitellogenic phase in oviparous vertebrates is characterized by intense RNA transcription (Davidson 1986) within the oocyte and some modest increase in size, concomitant with the growing follicle cell layers. The eventual fecundity, in rainbow trout, appears to be established at this time (Tyler et al. 1994). The bulk of oocyte enlargement in fishes and the largest overall increase in the size of the ovary occurs due to yolk accumulation during the vitellogenic phase. Both phases appear to be under gonadotropic regulation since hypophsectomy during the reproductive cycle inhibits further ovarian development and causes gonadal atrophy (Barr 1963). However, there is no evidence that the oocyte itself has gonadotropin receptors. The means by which gonadotropin signals detected by receptors in the follicle cells (Yan et al. 1992) are transmitted to the oocyte is not known. It is speculated that an endocrine factor is produced by the follicle cells, upon gonadotropin stimulation, which signals the oocyte.

The identification of the hormone(s) within the ovary that directly controls ovarian growth would be a huge step towards a means of monitoring development. Measurement of this endocrine agent could provide a way of categorizing females and identifying fish that will produce sub-fertile eggs. The ability to diagnose ovarian growth problems, possibly early during the pre-vitellogenic phase, might provide the fish manager with more time to remedy the situation. At the very least those fish could be removed from the breeding pool.

Of the two distinct gonadotropins (GTHs) in salmonids GTH I appears in the blood before GTH II (Prat et al. 1996) and is thought to be responsible for initiating and maintaining the ovarian growth phase (Tyler et al. 1997). The endocrine mechanism by which GTH I does this within the ovary is not known. The female sex steroid estradiol (E_2) is a probable candidate as an endocrine signaling agent within the salmonid ovary. This is due to the facts that ovarian synthesis of E_2 is stimulated and maintained by GTHs (Suzuki et al. 1988), and mRNAs for estrogen receptors have recently been detected in the ovary of the rainbow trout (Nagler and Cyr 1998). These estrogen receptor transcripts are present throughout reproductive development (Nagler, unpublished). This suggests that E_2, regulated by GTH I, may have an important function(s) within the ovary. The idea that E_2 has intra-ovarian biological effects is a departure from previous studies which show strictly extra-ovarian activities for this hormone in fishes. Indeed, one of the long established actions of E_2 on the liver of fishes is to stimulate the synthesis of vitellogenin. Vitellogenin is the major yolk precursor in fishes and it is endocytosed by receptors found on the surface of the oocyte (Prat et al. 1998). Since E_2 regulates the hepatic synthesis of vitellogenin it may also coordinate the synthesis or cycling of vitellogenin receptors within the oocyte. An impact of E_2 on ovarian development before the period of yolk deposition is also possible. The pre-vitellogenic ovarian follicle does undergo theca and granulosa cell proliferation and oocyte growth. Many studies in mammals show that E_2 has different and wide ranging effects within the ovary such as regulating levels of GTH receptor and stimulating growth factor synthesis (for review, see Richards 1994). The activity of E_2 in regard to these processes has not been explored in fish. It is possible that E_2 produced during early ovarian development may stimulate growth factors synthesis in follicle cells that, in turn, communicate with the oocyte. Both, insulin-like growth factor (IGF)-I and IGF-II have been demonstrated in the ovaries of rainbow trout and their nucleotide sequences are published (Chen et al. 1994). In carp (*Cyprinus carpio*), the ligands and receptors, for both insulin and IGF-I, have been demonstrated in the ovary (Maestro et al. 1997). Evidence from work with the killifish (*Fundulus heteroclitus*) indicate that IGFs can stimulate final oocyte maturation suggesting that the oocyte has IGF receptors on its cell surface (Negatu et al. 1998). These growth factors may be critical, not only for follicle cell layer growth, but also for oocyte development.

b. Rationale and significance to Regional Programs

The rationale of the present proposal is that developmental processes within the ovary leading up to ovulation will be critical for successful production of many, good quality eggs. One of the major difficulties in captive salmonid propagation programs is inconsistent embryo viability, which derives from problems during oocyte development and egg maturation. These circumstances are endangering wild salmonid populations in the Pacific Northwest by reducing the number of viable offspring produced by captive broodstock. It is particularly critical in populations where the number of quality eggs are limiting due to the few remaining females. The overall goal of this proposal is to study several novel endocrine processes within the developing salmonid ovary, to better understand ovarian development. **The general hypothesis is that an understanding of key endocrine processes within the developing salmonid ovary, particularly those involving estradiol, will lead to ways of diagnosing sub-fertility and improving reproductive success.** The significance of the proposed research is that endocrine events within the fish ovary that directly regulate oogenesis are not well known. Basic research on intra-ovarian processes will contribute to a better overall understanding of ovarian growth and development. This information will generate a basis from which new strategies and potential future technologies can be developed to enhance reproduction in captive salmonid broodstock programs.

c. Relationships to other projects

<u>Project 1</u>: The PIs from Projects 1 and 2 have mutual interests in fish reproductive biology, and have collaborated in the past on the maternal and paternal effects on embryo viability in rainbow trout. Since 1996 the PIs from both Projects have met for biweekly seminars of the WSU-UI Center for Reproductive Biology.

<u>Project 3:</u> The PIs from Project 2 and 3 have mutual interests in fish reproductive biology. Since 1996 the PIs from both Projects have met for biweekly seminars of the WSU-UI Center for Reproductive Biology.

<u>Project 4:</u> The PIs from Project 2 and 4 have similar interests in fish reproductive biology and specifically the involvement of IGFs in the ovarian development in salmonids. There will be mutual overlap between increased IGF levels expected upon growth hormone administration in Project 4 and the mechanism by which IGFs affect oocyte growth in Project 2. The development of IGF-I and -II radioimmunoassays are ongoing in Dr. Shelling's laboratory and it is planned to utilize these assays in Project 2 to quantify *in vitro* production by isolated ovarian follicles. PIs on Project 2 and 4 interact through activities generated by WSU-UI Center for Reproductive Biology.

<u>Project 5:</u> The PIs from Project 2 and 5 have mutual interests in salmonid reproductive biology and specifically characteristics of the egg that affect embryo viability. Studies proposed in Project 2 on ovarian development will compliment those on energy levels in eggs proposed in Project 5. Since 1996 the PIs from both Projects have met for biweekly seminars of the WSU-UI Center for Reproductive Biology.

<u>Project 6:</u> The PIs from Projects 2 and 6 have mutual interests in fish reproductive biology and interact through activities generated by WSU-UI Center for Reproductive Biology.

<u>Other Interactions:</u> For details please see Introduction section of the Fish Reproduction Program.

d. Project history (for ongoing projects)

None.

e. Proposal objectives

The overall goal of this investigation is to study several novel endocrine processes within the developing salmonid ovary, particularly those involving estradiol, to better understand ovarian development.

The specific objectives of this FY2000 project are as follows:

1) **To establish whether the principal female sex steroid, E_2, is the intra-ovarian endocrine factor responsible for regulating IGF production in the salmonid ovary.**
2) **Determine whether E_2, or hormones stimulated by E_2, influence the growth and development of pre-vitellogenic ovarian follicles.**
3) **Study whether E_2 can influence the transcription of genes for vitellogenin receptors within developing oocytes.**

This investigation proposes to examine previously unstudied endocrine pathways within the salmonid ovary as a means to understand normal ovarian growth, and provide an explanation for situations that lead to a sub-fertile condition. This information would be used to develop ways to diagnose sub-fertility (e.g. hormone test), and generate future technologies to improve reproductive success in valuable captive broodstock.

The completion of objectives for FY2000 will lead to the following objectives in subsequent years:

4) Investigate the possibility that the salmonid ovary contains mRNAs for kit ligand and/or its receptor, c-kit, and synthesizes these proteins.
5) Examine the controlling effects of temperature on growth factor synthesis and ovarian follicle growth.

f. Methods

General Procedures

Fish: Rainbow trout (*Oncorhynchus mykiss*) will be used for the experiments outlined in this proposal. These fish are available from numerous aquaculture facilities throughout the state on a year-round basis. Juvenile fish (i.e. 12" total length) can readily be purchased and raised to the desired state of sexual maturation at the UI Aquaculture Research Facility in Hagerman, ID. If fish smaller than 12" are required gametes can be purchased from Mt. Lassen Trout Farm (Red Bluff, CA) and are shipped Fed Ex for next day delivery. Artifical fertilization is carried out in our Departmental Wet Lab facility and the fertilized eggs incubated in a coldroom at $10^{o}C$ in a conventional vertical incubator (Heath, Tacoma, WA). Fish will be grown out in facilities to be built on the UI campus (Moscow, ID).

Data Analysis: An ANOVA ($P<0.05$) will be used to determine if significant differences exist within an experiment. In instances where multiple comparisons within an experiment are necessary Tukey's Test will be used to identify significant differences ($P<0.05$) between groups. All statistical analyses will be performed with SigmaStat software (Jandel Scientific Software, San Rafael, CA).

Experimental Design

Objective #1- Establish whether the principal female sex steroid, E_2, is the intra-ovarian endocrine factor responsible for regulating IGF production in the salmonid ovary.

Numerous studies in mammals show that E_2 can modulate ovarian growth factor synthesis (Hsu and Hammond 1990; Dorrington et al. 1993; Das et al. 1994). There is no information in fishes that similar actions of E_2 exist, although IGFs and their receptors have been reported in fish ovaries (Cao et al. 1989; Duguay et al. 1991; Maestro et al. 1997; Chen et al. 1994). Recently, IGFs were reported to stimulate fish oocyte final maturation suggesting that receptors exist on the oocyte cell surface for these growth factors (Negatu et al. 1998). **The hypothesis is that E_2 is capable of influencing the transcription of IFG-II mRNA and/or secretion of IGF-II by ovarian follicles of the rainbow trout.** IGF-II was chosen over IGF-I because the mRNA of the former is found in much higher levels than the latter in trout ovaries and IGF-I has 4 different forms in the trout whereas IGF-II has only one (Chen et al. 1994). The fact that only one form of IGF- II exists may initially be less complex to study. Further studies with IGF-I could be engaged in if IGF-II results show promise and/or the study of IGF-I becomes more relevant. Experimentation would involve *in vitro* ovarian follicle cultures from female trout with either pre-

vitellogenic or vitellogenic ovaries. Three replicates of five ovarian follicles from fish with vitellogenic ovaries or whole ovarian fragments from fish with pre-vitellogenic ovaries would be cultured for differing periods of time with graded amounts of E_2. Controls would be set up in identical fashion with no E_2 added. It will be necessary to determine the amount of time and E_2 dose empirically. Following incubation tissues would be frozen for later RNA extraction and media would be frozen for quantification of IGF protein by radioimmunoassay (RIA). The amount of IGF mRNA in ovarian follicles will be measured using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method. The method is based on co-amplification of a synthetic RNA (sRNA) internal standard with the same priming sites as the target mRNA (i.e. competitive RT-PCR) (Kagami et al. 1996). The sRNA will be engineered to be different in size, relative to the target mRNA, by insertion or deletion of DNA sequence between the priming sites. Primers will be designed based on the published rainbow trout IGF-II cDNA sequence (Shamblott and Chen, 1992). The size of the sRNA (e.g. 350 or 450) is designed to be different from the target mRNA (e.g. 400 bases) such that they can be resolved on an agarose gel. The assay involves isolating total RNA from tissue samples (1 mm^2) and adding a known amount of internal standard sRNA. Both transcripts are amplified by RT-PCR with the same primers, to reduce inter-tube variations, and a nucleotide labeled with [^{32}P] by RT-PCR. The PCR products are resolved on an agarose gel and the amount of radioactivity in each band determined with a phosphorimager. The amount of radioactivity in each unknown band would be compared to the linear portion of a curve of known quantities of internal standard amplified in each assay. Development would include establishing the reproducibility (i.e. intra-assay variability), specificity and sensitivity of the assay. The quantification of IGF protein secreted in the medium would be accomplished with homologous salmonid IGF RIAs, in collaboration with Dr. Gerald Shelling (a fellow PI involved in the Fish Reproduction Program, see Project #5) who presently has an IGF-I RIA operational in his laboratory. The development of a IGF-II RIA is planned using a procedure analogous to that for IGF-I.
<u>Potential Problems and Proposed Corrective Actions</u>**-** The *in vitro* incubation of trout gonads is not difficult and the PI has extensive experience in culturing trout gonadal fragments for short periods of time (i.e. 1 week) (Nagler et al. 1994; Nagler et al. 1996). The PI's laboratory recently developed a quantitative RT-PCR method to quantify estrogen receptor mRNA, therefore this technology will be applied to measuring IGF mRNA. In addition, numerous published techniques are available and currently being used in other laboratories within the WSU-UI Center for Reproductive Biology.
<u>Anticipated Results</u>**-** An increase or decrease in the ovarian mRNA and/or protein levels of IGF-II in E_2 treatments versus control treatments will support the hypothesis that E_2 can affect the transcription and/or secretion of IGF-II.

Objective #2- Determine whether E_2, or hormones stimulated by E_2, influence the growth and development of pre-vitellogenic ovarian follicles.

During the pre-vitellogenic growth phase within the trout ovary some follicle cell proliferation and oocyte enlargement occur. The increase in oocyte size is not due to yolk accumulation, but is thought to be under GTH regulation. The pre-vitellogenic trout ovary is capable of synthesizing E_2, via GTH, that could have growth related actions within the ovarian follicle. It is also possible (see Objective #1) that other endocrine factors are triggered by E_2 that themselves have significant growth promoting activities on somatic (i.e. thecal and granulosa cells) or germ (i.e. oocyte) cells. **The hypothesis is that the treatment of pre-vitellogenic ovarian follicles with E_2 will result in significant follicle enlargement due to growth of somatic or germ cells.** It is proposed to culture 5 ovarian follicles (< 0.4 mm) or ovarian fragments (50 mg), in triplicate, from pre-vitellogenic ovaries. The culture of large vitellogenic ovarian follicles for more than a few days has proved difficult and will not be attempted. The duration of culture, depending on survival, would vary from 1-6 days and be determined empirically. Ovarian follicles would be treated with graded amounts of E_2 or culture media from previous incubations (Objective #1) that have been stripped of steroids by charcoal treatment. The steroid free media from earlier incubations would be used to test whether unknown protein factors produced by follicles under E_2 treatment can enhance growth. Controls will be treated in an identical fashion but without E_2, or media treated with E_2. Ovarian follicle growth would be quantified in two ways, a) directly measuring individual follicle diameter, and b) by measuring the amount of radioactive thymidine incorporation. Radioactive thymidine would be added to the media at the start of culture.
<u>Potential Problems and Proposed Corrective Actions</u>**-** Similar to Objective #1, the *in vitro* incubation of trout gonads is not difficult and the PI has extensive experience in culturing trout ovarian follicles.

Anticipated Results- Significant increases in the diameter of ovarian follicles or enhanced radioactive thymidine incorporation under E_2 treatment as opposed to controls will support the hypothesis. It is possible that media stripped of steroids (i.e. E_2) may enhanced ovarian follicle growth. This would indicate that some unknown factor(s) is being stimulated by E_2 treatment and released into the medium that promotes growth.

Objective #3- Study whether E_2 can influence the transcription of genes for vitellogenin receptors within developing oocytes.

The vitellogenic phase of ovarian development in fish is characterized by yolk deposition in the cytoplasm of the oocyte. Vitellogenin (VTG) produced in the liver and secreted into the blood stream is acquired by the oocyte via receptors embedded in the oocyte plasma membrane (Wallace 1985). These VTG receptors operate by endocytosis, after which VTG is released from the receptor, and cleaved into the yolk proteins lipovitellin and phosvitin. The receptor is then cycled back to the oocyte surface. Recently, the cDNA sequences for two different VTG receptors was reported in the rainbow trout (Prat et al. 1998). Since E_2 stimulates the hepatic synthesis of VTG it may also prepare the oocyte to receive VTG by initiating or upregulating VTG receptor transcription. **The hypothesis to be tested is that E_2 can upregulate the transcription of one or both of the reported VTG receptors in developing trout ovaries.** It is proposed to culture pre-vitellogenic ovarian follicles either with E_2 or without, and determine whether VTG receptor transcription is altered. Pre-vitellogenic ovaries would be used since VTG receptor levels have been shown to be lower during this phase, than later on in vitellogenic ovaries (Tyler and Lancaster 1993). Any increase in transcriptional levels should therefore be more detectable in pre-vitellogenic ovaries. The levels of VTG receptor would be measured by Northern blot analysis (described in Nagler and Cyr, 1997). The DNA sequences for two rainbow trout VTG receptors are known (Prat et al. 1998) and would be used to design probes.
Potential Problems and Proposed Corrective Actions- No problems with this Objective are envisioned. Northern blot analysis is routine in the PIs laboratory and rainbow trout VTG receptor DNA sequences are published that could be used for probe design.
Anticipated Results- The experiments outlined above will show that E_2 can upregulate the transcription of trout VTG receptors and support the hypothesis proposed.

Beyond FY2000:

Objective #4- Investigate the possibility that the salmonid ovary contains mRNAs for kit ligand and/or its receptor, c-kit, and synthesizes these proteins.

The possibility that kit-ligand could be an important, but as yet unstudied, growth factor in the salmonid ovary is put forth because of it's well documented role in the growth of mammalian oocytes (Manova et al. 1993; Packer et al. 1994). **The hypothesis is that kit-ligand and/or its receptor c-kit is produced by the salmonid ovarian follicle.** A RT-PCR based approach would be used with degenerate primers based on mammalian kit-ligand sequences and mRNA from trout ovaries. PCR products of the expected size would be sequenced and compared to databases (e.g. GenBank) to determine their identity. Sequences with high similarity to established kit-ligands would subsequently be used to screen a trout ovarian cDNA library. Both immature and mature rainbow trout ovary cDNA libraries are available from Dr. Joe Cloud's (a fellow PI in this Fish Reproduction Program) laboratory for screening. Bacterial colonies hybridizing with the DNA probes would be isolated, plasmid DNA prepared, and sequenced to confirm their identity. Once a kit-ligand cDNA is obtained *in situ* hybridization experiments would be conducted to localize where in the ovarian follicle the mRNA is found. Short peptides (15-20 amino acids) will be synthesized using the deduced amino acid sequence and utilized to produce a specific antibody (Research Genetics, Huntsville, AL). Antibodies would permit immunocytochemical detection of kit-ligand proteins in the ovary and quantification would employ Western blots of the media to assess the degree kit-ligand is being secreted by ovarian follicles at different stages of reproductive development. A similar approach would be used to uncover the kit-ligand receptor, c-kit.
Potential Problems and Proposed Corrective Actions- The isolation of rainbow trout kit-ligand and/or c-kit receptor cDNAs could prove difficult if RT-PCR fails to amplify useful DNA fragments. This is an

acknowledged pitfall of this approach. An alternative approach would be to use kit-ligand DNA probes from other animals (e.g. mammals) to probe a rainbow trout ovary cDNA library.
Anticipated Results- A rainbow trout kit-ligand and/or c-kit receptor cDNA(s) will be isolated upon screening an ovarian cDNA library. This will confirm the hypothesis that kit-ligand and/or c-kit receptor is present in the rainbow trout ovary. The cDNA sequences will be used as probes to localize the cell type producing these transcripts and to deduce enough amino acid sequence to generate an immunogen for antisera production. The levels of ovarian kit-ligand and c-kit receptor protein produced by ovarian follicles at different stages of reproductive development will be quantified.

Objective #5- Examine the controlling effects of temperature on growth factor synthesis and ovarian follicle growth.

The detrimental effects of temperature extremes on reproductive maturation of salmonids is well known (for review, see Swanson et al. 1995). Interestingly, the upper temperature threshold at which reproduction is inhibited is much lower than the upper lethal temperature. This suggests that a restricted temperature preferendum for reproduction exists in salmonids well within the extreme temperature limits for survival. What is not clear is whether temperature effects on reproduction occur at the level of the brain, pituitary or gonad. Based on peripheral blood endocrine profiles there are no differences between maturing fish held at different temperatures (Swanson et al. 1995), lending support to the idea that temperature may be acting specifically on intra-ovarian physiology. It is known that heat shock proteins exist in salmonid gonads (Zafarullah et al. 1992), and that these proteins can interact with ligand-steroid hormone receptor complexes and their binding to response elements (Pratt and Scherrer 1993). The most notable effect of heat shock proteins would be alterations in the transcription of steroid hormone responsive genes. Key endocrine pathways involving steroids (e.g. E_2) in the ovaries may be affected by mild hyperthermia via heat shock proteins. **The hypothesis to be tested is that a reproductive temperature preferendum exists for salmonids, well within the extreme temperature limits for survival and that the basis for this limit is defined by endocrine processes within the ovary.** Experiments will be conducted that examine the effect of increasing temperatures on the transcription of growth factors (e.g. IGF and kit-ligand) and VTG mRNAs. These experiments will build on the success and directions generated in Objectives #1-4 above. Three replicates of five ovarian follicles or whole ovarian fragments from fish with previtellogenic ovaries would be cultured at selected temperatures (12, 14, 16, 18^{o}C). Following incubation tissues and media would be frozen. It is proposed to measure IGF and kit-ligand mRNAs by qRT-PCR to determine whether transcription rates have been altered by the incubation temperature. Kit-ligand mRNA would be measured by quantitative RT-PCR similar to that proposed for IGF in Objective #1. The VTG mRNAs would be measured by Northern blotting as described in Objective #3.
Potential Problems and Proposed Corrective Actions- No problems with quantification of IGF mRNAs by qRT-PCR are envisioned. The measurement of kit-ligand mRNA would be contingent on isolation of the cDNA for this growth factor from trout ovaries as described in Objective #4. Northern blot analysis is routine in the PIs laboratory and rainbow trout VTG receptor DNA sequences are published that could be used for probe design.
Anticipated Results- The experiments outlined will show that certain temperatures can downregulate the transcription of trout ovarian growth factors and/or VTG receptor mRNAs supporting the hypothesis proposed.

g. Facilities and equipment

The principal investigator has a 600 sq. ft research laboratory located in Gibb Hall (Rm 235) with typical standard equipment (fume hood, micro and top-loading balances, pH meter, water bath, microfuges and hand held pipettors). Specialized items include a Micron cryostat, Zeiss microscope and stereomicroscope, Thermolyne hybridization oven, Perkin-Elmer PCR thermal cycler, Beckman refrigerated centrifuge, BioRad submarine and vertical gel electrophoresis equipment, Nuaire Class II laminar flow hood, NapCo CO_2 incubator, Shimadzu UV spectrophotometer and Biomark PIT electronic tag reader. The adjacent Room 236 is a Wet Lab with three living stream units and a cold room containing two eight-tray Heath incubation units.

Common use equipment such as phosphoimager, ultracentrifuges, liquid scintillation and gamma counters, and speed vac concentrator are generally available within the Department of Biological Sciences. Fish holding facilities, suitable for raising and maintaining large numbers of rainbow trout, are available at the University of Idaho fish hatchery in Hagerman, ID. These facilities encompass both small and large isolated tanks, and raceway systems supplied with a high quality single pass water supply at 15^{o}C.

The PI is a member of the Center for Reproductive Biology, an inter-university organization which formed between Washington State University and the University of Idaho in 1996. The Core Facilities of the Center for Reproductive Biology (Molecular Biology, Radioimmunoassay and Histology) would be available.

h. Budget

Personnel:
Summer salary (2.0 months per year) is requested for the principal investigator, Dr. James Nagler, Assistant Professor of Zoology, UI. During this two month period the PI will be devoting 100% of his time to the proposed project. The PI will be responsible for designing experiments, supervision of the laboratory research, analysis of the data, and writing progress reports and manuscripts. Full support for a research technician ($34,000) and postdoctoral fellow ($30,000) is requested. The technician and fellow would be devoted 100% of the time to the proposed research. These persons would be responsible for conducting experiments, sample analysis, fish husbandry, data presentation, and general laboratories duties (making solutions, cleaning, glassware, equipment maintenance). The fringe benefits set for faculty and staff on salary >$30,000 at UI is 28.5%. Salaries and fringe benefits have been increased by 5% for each of the outyear budget estimates.

Supplies:
The funds budgeted ($13,000) will be used to purchase consumables and reagents for molecular and histological work; these items will include molecular biology kits, restriction enzymes and polymerases, buffer chemicals, consumables [plasticware, pipet tips, centrifuge tubes], and histological supplies. The request also includes the cost of rainbow trout and the associated supplies for animal care (e.g. food).

Operations and maintenance:
Funds are needed to cover transportation costs ($1,500) to obtain fish (e.g. trucking) or ship gametes (e.g. air freight). Funds are budgeted ($1,000) for publication-related activities (e.g. photocopying, digital imaging, page charges). The user fees for wet lab facilities (e.g. water and space charges) at the University of Idaho Aquaculture Research Institute will total about $1,000. The Department of Biological Sciences, UI levies an equipment charge to cover maintenance on common use departmental equipment amounting to $1,500.

Capital Acquisitions:
The cost ($10,000) associated with equipment (i.e. tanks, water chillers, recirculating pumps) needed for construction of appropriate fish holding facilities on the UI campus (Moscow, ID) is requested. These on-campus facilities are needed to conduct the proposed work and are being requested in the first year only.

Travel:
The travel funds requested ($2,000) will be used by the PI to attend one national meeting per year. Examples of meetings include Society for the Study of Reproduction and American Fisheries Society Annual General Meetings. Travel funds have been increased by 5% for each of the outyear budget estimates.

Other:
Administrative Core- This Core laboratory will integrate the activities of the Fish Reproduction Program and the Center for Reproductive Biology for administration of the grants, core laboratories, and activities such as seminars, workshops, and retreats. The integration of the different projects and research activities requires the Administrative Core laboratory support requested.

Hormone Assay Core- This Core laboratory provides the service of examining reproductive hormone levels in various fish biological fluids (e.g. blood). This is a critical component of the proposed research. The support requested is for the expenditures related to this Core laboratory.
Aquaculture Core- This Core laboratory provides the faculty and services required for the maintenance of fish populations for the proposed research. This is a critical aspect of the proposed studies. The support requested is for the expenditures related to this Core laboratory.

Section 9. Key personnel

Principal Investigator: James J. Nagler

1. Present Position:

Assistant Professor of Zoology (1996- present)
Department of Biological Sciences,
University of Idaho
Moscow, ID 83844-3051

2. Education:

i) Ph.D. (Fish Reproductive Physiology)
Memorial University of Newfoundland, Canada (1991).

ii) M.Sc. (Fish Reproductive Toxicology)
Concordia University, Canada (1985).

iii) B.Sc. (Fish and Wildlife Biology)
University of Guelph, Canada (1983).

3. Recent employment history:

i) Visiting Scientist: Marine Environmental Sciences Division, Maurice Lamontagne Institute, Fisheries and Oceans, Mont-Joli, QC, Canada (August 1994- June 1996).
ii) Postdoctoral Fellow: Fish Physiology Research Group, Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UK (August 1992- July 1994).
iii) Postdoctoral Fellow: Ocean Sciences Centre/ MSRL, Memorial University of Newfoundland St. John's, NF, Canada (October 1991- June 1992).

4. Relevant publications:

Nagler, James J., Alexander P. Scott, Charles Tyler and John P. Sumpter (1996) Gonadotropins I and II do not stimulate the *in vitro* secretion of 17α,20β -dihydroxy-4-pregnen-3-one 20-sulphate by rainbow trout gonads during final sexual maturation. Fish Physiol. Biochem. 15: 149-156.
Tyler, Charles R., Tom G. Pottinger, E. Santos, John P. Sumpter, S-A. Price, Suzanne Brooks and James J. Nagler (1996); Mechanisms controlling egg size and number in the rainbow trout *Oncorhynchus mykiss*. Biol. Reprod. 54:8-15.
Tyler, Charles R., James J. Nagler, Tom G. Pottinger and Mark A. Turner (1994); Effects of unilateral ovariectomy on recruitment and growth of follicles in the rainbow trout, *Oncorhynchus mykiss*. Fish Physiol. Biochem. 13:309-316.

Nagler, James J., Charles R. Tyler and John P. Sumpter (1994); Ovarian follicles of rainbow trout *(Oncorhynchus mykiss*) cultured within lamellae survive well, and sequester and process vitellogenin. J. Exp. Zool. 269:45-52.

Nagler, James J., Faye Murrin and David R. Idler (1993); Localization of vitellogenin -related protein in the ovarian follicle of winter flounder (*Pleuronectes americanus*) by protein A-gold immunocytochemistry. Cell Tissue Res. 271: 567 -570.

Section 10. Information/technology transfer

The results obtained will be published in peer-reviewed scientific journals and presented at scientific conferences. A summary of major findings will be presented as progress reports to BPA annually.

Congratulations!